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Agriculture Development Fund

FINAL REPORT

DEVELOPMENT OF WAXY WHEAT

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FINAL REPORT

Development of Waxy Wheat

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1 ABSTRACT

Wheat grains with waxy endosperm starch contain low amylose and high amylopectin concentrations. Fully and partially waxy wheat starches have unique food (noodles) and industrial uses, which can result in crop diversification and value-added processing in Saskatchewan. Granule-bound starch synthase 1 (GBSS1) is a key enzyme that synthesizes amylose. We have produced antibodies against GBSS1 for use in developing immunochemical techniques to study and characterize GBSS1 in plant starch granules. A high-performance liquid chromatography (HPLC) based technique was optimized to precisely measure amylose concentration in wheat starch. A wheat line that lacked GBSS1 B and D isoprotein was identified and characterized. We have developed adapted, fully waxy (>1% amylose) wheat lines for potential cultivar release. Partially waxy wheat lines (≅ 20-21% amylose), with Udon noodle making qualities, have also been produced.

2 EXECUTIVE SUMMARY

The objective of this project is to develop wheat cultivars with waxy endosperm starch (low amylose and high amylopectin concentrations). Wheat grain with low amylose endosperm starch is preferred for the production of 'Udon' noodles in Japan and the Pacific-rim countries. Canadian wheat cultivars currently in use lack the starch characteristics needed for making good 'Udon' noodles. The waxy locus (*wx*) encodes granule-bound starch synthase 1 (GBSS1), a key enzyme responsible for amylose synthesis. A reduction in GBSS1 activity results in decreased amylose concentration in the grain. The objectives of this research project were: (i) to develop biochemical and molecular tools to study and assay the waxy gene and its products, and (ii) to produce partially waxy and/or waxy wheat homozygous lines.

(i) Biochemical and Molecular Techniques to Assay and Characterize the Waxy Gene and its Products:

(a) The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique was modified to separate the GBSS1 Band D genome products. The improved SDS-PAGE resulted in increased discernment between the B and D genome products, and the observation that almost forty percent of current Canadian wheat cultivars lack the GBSS1 B isoprotein.

(b) A HPLC technique was optimized to precisely determine amylose concentration in wheat starch. The HPLC technique showed that the fully waxy wheat starch did not have detectable amylose.

(c) Antibodies against the GBSS1 D isoprotein were produced. The immunoblot analysis revealed that GBSS1 from all the wheat genomes, as well as from corn, oat, rice barley, pea and potato, are immunologically related.

(ii) To produce partially waxy and fully waxy wheat homozygous lines.

(a) Developed fully waxy homozygous lines for cultivar registration purposes.

(b) Developed partially waxy lines with Udon noodle-making qualities.

(iii) Characterization of partially waxy and fully-waxy wheat starch.

(a) Absence of GBSS1 B and D isoproteins reduced the amylose concentration in wheat endosperm starch to 12%.

(b) Starch with reduced amylose concentration has higher crystallinity, higher enthalpies of gelatinization and increased transition temperature, as compared to wheat starch with normal amylose (25%) concentration.

3 INTRODUCTION

Starch granules of plants contain two types of glucan polymers: a predominantly linear amylose and a highly branched amylopectin. The ratio of amylose to amylopectin determines the physico-chemical properties of starch and, thereby, the end-use of the grain. Starch biosynthesis in plants is accomplished by the action of several enzymes that include adenosine glucose pyrophosphorylase, starch synthases, branching and debranching enzymes (Preiss and Sivak 1998). One of the main starch biosynthetic enzymes is granule bound starch synthase1 (GBSS1, E.C.2.4.1.21), which is a key enzyme in amylose biosynthesis (Echt and Schwartz 1981). In wheat, GBSS1 is termed waxy protein (Wx) and is encoded by the waxy locus (*wx*) located on chromosomes 7AS, 4AL and 7DS (Chao et al 1989). Mutations at all three *wx* loci result in waxy wheat starch that is predominantly composed of amylopectin (Nakamura et al 1995). Chemically modified waxy wheat grain starch may find applications in the food industry similar to those identified for waxy maize starch (Reddy and Seib 2000). A mutation at the GBSS1 B locus results in a slightly reduced amylose concentration in endosperm starch and better noodle making quality than is the case for wild wheat (Miura and Tanii 1994, Zhao et al 1998). There is also a strong correlation between the GBSS1 B mutation and flour swelling volume (Zhao et al 1998), and a reduction in amylose concentration also is associated with a higher amylogram peak viscosity (Zeng et al 1997).

Mutations at the waxy loci in wheat have been reported by various groups (Demeke et al 1997, Graybosch et al 1998, Yamamori et al 1994, Zhao et al 1998). Purification and separation of GBSS1 from more than 200 North American hexaploid wheats has resulted in the identification of seven wheat lines with null alleles for GBSS1

A and 13 with null alleles for GBSS1 B, and one double mutant for GBSS1 A and B isoproteins (Graybosch et al 1998). However, none of the characterizations identified a wheat line carrying mutations at both B and D GBSS1 loci. The absence of individual GBSS1 isoproteins slightly reduces amylose concentration (Fujita et al 1998). The effect of different GBSS1 double mutants on amylose production and starch characteristics is not documented in the literature (Zhao et al 1998).

4 OBJECTIVES

The overall objective of this research are to develop wheat cultivars with waxy and partially waxy endosperm starch. The specific objectives for this research project are:

A. Develop biochemical and molecular tools to precisely characterize waxy gene and assay its products.

B. Produce partially waxy and/or waxy wheat homozygous experimental lines.

5 MATERIALS AND METHODS

5.1 Seed Sources

Grains of various wheat genotypes were obtained from the USDA germplasm collection center at Aberdeen, Idaho, USA. Grains of the Canadian and U.S. cultivars were kindly provided by the Canadian Grain Commission.

5.2.1 Starch Granule Isolation

Starch granules were isolated from embryo-less half seed(s) according to the procedure described by Zhao and Sharp (1994, 1996) and Demeke et al., (1997, 1999). In a microfuge tube (1.5 mL) embryo-less single seeds were soaked in 1 mL of double distilled water and kept at 4°C overnight. After centrifugation at 15,000 x g for five minutes, the supernatant was removed and the pellet was crushed in 300 µL of double distilled water using a plastic pestle. The slurry was over-layered on 1 mL of 80% cesium chloride and centrifuged at 15,000 x g for 5 min. The starch granule pellet was washed twice with 1 mL of wash buffer (55 mM Tris-HCl, pH 6.8; 2.3% SDS, w/v; 5% ß-mercaptoethanol, v/v; and 10% glycerol, v/v), three times with water and once with acetone. The samples were centrifuged at 15,000 x g for three minutes after each washing step.

5.3 GBSS 1 Protein Isolation, SDS-PAGE, and N-Terminal Protein Sequencing

Proteins from the starch granules were extracted with 100 µL of buffer (62.5 mM Tris-HCl, pH 6.8; 4% SDS, w/v; 5% ß-mercaptoethanol, v/v; and 10% glycerol, v/v) boiled for 5 min, and then cooled on ice for about 10 min. The cooled starch suspension was centrifuged, and the supernatant analyzed by gel-electrophoresis after the addition of 0.005% (w/v) bromophenol blue. GBSS1 proteins were extracted and analyzed from at

least three individual kernels for each cultivar and from more than three kernels, when the results were inconclusive. For one-dimensional gel-electrophoresis, the resolving gel contained 20% of 30:0.1% acrylamide/bisacrylamide, w/v; 375 mM Tris-HCl, pH 7.8; 0.1% SDS, w/v; 0.05% ammonium persulphate, w/v; and 0.05% TEMED, v/v. The stacking gel contained 126 mM Tris-HCl, pH 6.8; 4% of 30/0.8% acrylamide/ bisacrylamide, w/v; 0.1% SDS, w/v; 0.05% ammonium persulphate, w/v; and 0.05% TEMED, v/v. The running buffer contained 3.03 g Tris-base, 40 g glycine and 1.0 g SDS per liter of double distilled water (no pH adjustment). The gel was pre-run for 1.5 h at 15 mA per gel (0.75 mM thick gel, Protean II BioRad apparatus). Protein samples were electrophoresed for at least 20 h at a constant current of 15 mA per gel at 14 ± 1°C . Polypeptides separated on gels were visualized by silver-staining (Silver Stain Plus, BioRad).

The procedure for two-dimensional gel electrophoresis was adapted from O'Farrel (1975) and Nakamura et al., (1993). Ten mg of starch was dissolved in lysis buffer (8M urea; 2% v/v Nonidet P-40 [NP-40]; 2% v/v ampholine, pH 3.5-10; 5% v/v β-mercaptoethanol; and 0.05-5% w/v insoluble polyvinylpolypyrrolidone [PVPP]. The starch dissolved in lysis buffer was either heated at 40-60°C for 10 min or boiled for 2 min, cooled on ice, and centrifuged at 15,000xg for 12 min. The supernatant (50-70 μL) was loaded on an isoelectric focusing gel tube (1.5 mm x 18 cm). The isoelectric focusing gel consisted of 3.5% (w/v) acrylamide-bisacrylamide, 2.5% (v/v) ampholines, pH 3.5-10 and ph 5-8, to give a range of pH 4.5-8; 8M urea; 2% (v/v) NP-40; 0.1% (v/v) TEMED; and 10% (w/v) ammonium persulphate. The proteins in isoelectric focusing gel were electrphoresed at 400V for 15-17 hr and finally at 800 V for 1 hr. The temperature

during electrphoresis was maintained at 14±1°C. The acidic running buffer was 0.01M H_3PO_4, and the basic running buffer was 0.02M NaOH. The proteins separated by isoelectric focusing were then separated by SDS-PAGE as described above.

For N-termina! sequencing of the GBSS 1 isoproteins, the separated GBSS 1 polypeptides were transferred to a PVDF membrane (Millipore, Bedford, MA, USA) by electro-blotting and visualised on the membranes by Coomassie blue staining. The pieces of immobilon blots carrying GBSS1 isoproteins were placed in the sample cartridge (Matsudaira 1987) of a Porton 2090E gas-phase protein sequencer (Beckman, Fullerton, CA USA) equipped with an on-line Hewlett Packard 1090L HPLC for amino acid sequencing.

5.4 Preparation of GBSS Antibodies

For producing antibodies, the GBSS 1 isoprotein was isolated from starch granules of a wheat line that has the null alleles for A and B isoproteins (has only the GBSS 1D isoprotein). The starch granule proteins were resolved on SDS-PAGE as described above. Location of GBSS 1 was determined based on its relative migration. Electroeluted GBSS 1 proteins were concentrated by micro-concentrators (Centricon with 30,000 MW cutoff; Amicon Canada Ltd.) lyophilized, and dissolved in phosphate buffered saline to be injected into a cereal-starved rabbit to produce anti-serum as described (Båga et al., 2000). The homogeneity of the isolated GBSS 1D was verified by two-dimensional SDS-PAGE.

5.5 Immunoblot Analysis

The starch granule proteins extracted as above were electrophoresed on a 5-12% gradient SDS-PAGE (0.75 mm thick) and transferred electrophoretically to a nitrocellulose

membrane. Immunoblots were incubated with the GBSS 1D antibody at a dilution og 1:100 for 3 hr and the immunoreactive bands were identified by chemical staining with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium (Stratgene, USA) following suppliers instructions.

5.6 Determination of Amylose Concentration

(a) *Megazyme Method*: Amylose concentration was determined according to the method of Gibson et al (1997) using the Megazyme Assay Kit (Megazyme International, Ireland). Wheat kernels were milled in a UDY cyclone mill (UDY Corporation, Fort Collins, CO, USA) and sifted through a 0.5 mm sieve. A 25 mg sample of the flour was boiled in 1 mL of dimethyl sulfoxide (DMSO) for 15 min. Ethanol (95%) was added to the boiled solution to precipitate and defat the starch. The defatted starch pellet was redissolved in 1 mL DMSO and diluted to 25 mL with 30% Con A solvent. Concentrated Con A solvent contained 0.52 M Sodium acetate, 3.0 M NaCl, 3.4 mM $CaCl_2$. 2H2O, 3.44 mM $MgCl_2.6H_2O$, and 3.54 mM $MnCl_2.4H_2O$, pH 6.4. A 0.5 mL aliquot taken from the 25 mL of solution was used to determine the total starch content by the amyloglucosidase/α-amylase method (Gibson et al 1997). Concanavalin A (Con A) was added to a 1.0 mL aliquot taken from the 25 mL of solution, to precipitate amylopectin. The amylose in the supernatant was hydrolyzed to glucose by amyloglucosidase/α-amylase, and the amylose concentration was expressed as a percentage of the total starch content. Three replicates of each starch samples were used to calculate Analysis of Variance using MINITAB for Windows Computer Software (1994).

(b) *HPLC Method*: To determine the amylose concentration by high performance size exclusion chromatography (HPSEC), a 5-mg starch sample was suspended in 5 mL

of double distilled-water in a glass tube and incubated at 130°C for 30 min. To 1 mL of the vortexed starch solution, 55 μL of 1M sodium acetate, pH 4.0, was added. The solution was vigorously mixed, and four units of isoamylase (200 units/mL stock solution, Megazyme) were added to debranch the starch. After 4 h of incubation at 40°C, the reaction mixture was boiled for 20 min to inactivate the isoamylase, and the starch solution was freeze-dried. The debranched starch was dissolved in 200 μL of DMSO solution (99% DMSO and 1% nano pure water), and centrifuged at 15000 x g for 10 min. Forty microliters of the supernatant was injected into a PLgel 5 μM MiniMix-C guard column attached to a PLgel MiniMix 4.6-mm ID column (Polymer Laboratories, Inc., Amherst, Massachusetts, U.S.A.) to separate amylose and amylopectin using a high performance liquid chromatography system (Waters 600 Controller, Waters 610 Fluid Unit, Waters 717 plus Autosampler, Waters 410 Differential Refractometer). The data were collected and analyzed using Millenium 2010™ Chromatography Software. Starch samples, column and detector were maintained at 40°C, 100°C and 45°C, respectively. Dimethyl Sulphoxide (99%) was used as an eluent at a flow rate of 0.2 mL/min. The amylose concentration of the starch samples was calculated by integration of the peak area corresponding to amylose, to that of the peak area corresponding to both amylose and amylopectin.

5.7 Analysis of Starch by Differential Scanning Calorimeter (DSC)

The thermal characteristics of starch samples were measured by a DSC (Mettler, TA3000). A 4-5 mg starch sample was mixed with water in a DSC pan to give a water-volume fraction of 0.77, based on the total volume of starch (density 1.5). The sample was heated at 10°C min^{-1} and the transition temperatures were recorded from a plot of

heat flow versus temperature (35-120°C). The enthalpy of gelatinization (ΔH) was calculated by measuring the curve area and is reported in J/g. The onset (T_o), peak (T_P) and end of transition or conclusion (T_c) temperatures were taken from a plot of the relationship between differential (slope) and temperature. The differential was computed from a relationship between heat flow and temperature during starch gelatinization. Two determinations of three sets were analyzed for each wheat starch sample and the results are expressed as the mean of six determinations.

5.8 Analysis of Starch by X-Ray Diffraction

Reagent grade alumina powder (Anachemia, 200 mesh Al_2O_3) was used as an internal reference standard (reference peak at 38.50°, 2θ) for measuring the relative intensity of starch diffraction peaks and providing an absolute diffraction angle (2θ) reference position. A 1:10 mixture of alumina and starch (w/w) was densely packed into an aluminum sample holder. The X-ray diffraction pattern (diffractogram) of the starch sample was recorded on a Phillips Model 42273 diffractometer with a Phillips PW-1965-60 proportional detector and an Enraf-Nonius FR590 2.0 kVA X-ray generator. The sample was scanned from 3° to 40° 2θ at an equivalent angular velocity of 0.6° 2θ/min, using a step interval of 0.02° 2θ, a count time of 1.2 sec and Cu Kα radiation (1.54178Å) at 1.6 kVA. The d-spacing was calculated from 2θ according to Bragg's equation ($n\lambda = 2d \sin\theta$; where d = intercrystalline spacing, n = 1, and λ = 1.54178Å). The relative intensities of the major diffraction peaks of starch were calculated as the ratio between the absolute intensity of the starch peak and the reference peak.

6 RESULTS AND DISCUSSION

6.1 Frequent Absence of GBSS1 B Isoprotein in Canadian Wheat Cultivars

A typical separation of the three GBSS1 isoproteins is depicted in Fig.1. Pre-running the SDS-PAGE gel for 1.5 h before applying samples was essential to separate GBSS1 B and D isoproteins. The gel running buffer also had a large amount of glycine (40g/L) as compared to regular SDS-PAGE protocols. Meticulous care is required in the SDS-PAGE procedure to obtain reproducible results. Of the Canadian wheat cultivars assessed, 46 had the three GBSS1 isoproteins, whereas 40 did not have the GBSS1 B isoprotein in their endosperm starch granules (Table 1). The cv Lake was a mixture, as some seeds had all three GBSS1 isoproteins, whereas some seeds lacked the GBSS1 B isoprotein in their endosperm starch granules. Rodríguez-Quijano et al. (1998) and Yamamori and Quynh (2000) have recently reported a variant for GBSS1 B isoprotein. The most common mutation is designated as *Wx-B1b* (GBSS1 null B) and the new variant is designated as *Wx-B1c'* (Rodriguez-Quijano et al. (1998) or *Wx-B1e* (Yamamori and Quynh, 2000). The *Wx-B1e* variant is reported to migrate slower than the normal GBSS1 B isoprotein, and is not as frequently observed as the *Wx-B1b* variant. We did not distinguish between the GBSS 1 B isoprotein encoded by the *Wx-B1b* and the recently identified variant allele. However, we have also observed a relatively thicker lower GBSS1 band (e.g., AC Minto, AC Barrie), similar to that shown in Fig. 1 of Yamamori and Quynh (2000), which may correspond to *Wx-B1e*, but separation of the two bands was not achieved by running the SDS-PAGE gel for over 22 hours. The separation is also inconclusive as it depends on the size and type of gel used. For example, Yamamori and Quynh (2000) reported that the SDS-PAGE gel they used was

smaller in dimension than that of Zhao and Sharp (1996) and thus they were not able to distinguish the GBSS 1 B isoproteins encoded by *Wx-B1e* or *Wx-D1a*.

About 46% of the Canadian wheat cultivars surveyed did not have the GBSS1 B isoprotein (Table 1). Most of the mutations at GBSS1 B locus have been reported in Australian wheat cultivars (Zhao et al. 1998; Yamamori et al. 1994). Yamamori et al.(1994) reported that 40% of the 127 Australian wheat cultivars studied lacked the GBBS 1 B isoprotein. In a recent study, Rodríguez-Quijano et al. (1998) reported 17% of the Iberian hexaploid wheats also did not have the GBSS1 B isoprotein, which suggests a common occurrence of mutation at the GBSS1 B locus. Canadian wheat cultivars have a narrow genetic base, and many of the cultivars lacking the GBSS1 B isoprotein have a common ancestry. The Canada Western Red Spring (CWRS) wheat cultivars have become progressively more interrelated based on a Thatcher background (van Beuningen and Busch, 1997a, b). The cultivar Thatcher is null B for GBSS1 isoprotein, and cultivars that are closely related to Thatcher in their pedigree, such as Canthatch, Saunders, Pembina, Manitou and others (Table 1), are also null B for GBSS1. The cultivars Conway, Laura and Invader are less reliant on Thatcher for their parentage; however, the latter is still one of the largest contributors to their pedigrees. The recently registered cultivars, McKenzie (Columbus/Amidon) and Prodigy (Columbus/ Kenyon sib//Stoa) both have parents that have a mutation at the GBSS1 B locus. We also observed that the US spring wheat cultivars Stoa and Amidon both lack the GBSS1 B isoprotein. Thatcher is a US cultivar released in 1934 and has the pedigree of Marquis/Iumillo//Marquis/Kanred. The cultivar Marquis (Hard Red Calcutta/Red Fife) and its parents have all three GBSS1 isoproteins. Iumillo is a durum wheat. We have not

tested Kanred (hard red wheat selected from "Crimean" group of winter wheats), which may be null B for GBSS1.

Based on a survey of the origin of Canadian wheat cultivars, Neepawa and HY320 were the most commonly used parents of cultivars released from 1981-1991 (Mercado et al. 1996). In this study, cultivars that have Neepawa in their pedigree, such as AC Michael, AC Barrie, and Columbus are null B for GBSS1 (Table 1). Mercado et al. (1996) also clustered AC Minto, Columbus, Katepwa, Kenyon and Pasqua in one group based on pedigree similarity and all are null B for GBSS1 isoprotein. AC Taber, Biggar, Genesis and HY320 were grouped as a cluster based on pedigree similarity (Mercado et al. 1996), and all are normal for GBSS1 isoproteins. Similarly, the strong gluten cultivars Bluesky, Glenlea, and Wildcat were clustered in one group, and these along with Laser belonging to the Canada Western Extra Strong market class, all have three GBSS1 isoproteins (Table 1). The cultivar Lake was a mixture as it contained some seeds with all three GBSS1 isoproteins and some seeds that lacked the GBSS1 B isoprotein. The cultivar Lake has Regent (GBSS1 null B) in its pedigree, so it could have a mutation at the GBSS1 B locus.

In summary, nearly half of the Canadian wheat cultivars studied did not have the GBSS1 B isoprotein. This frequency of GBSS1 B absence is comparable to that of the Australian wheat cultivars, which have high frequency of mutation GBSS1 B locus. The high frequency of GBSS1 B isoprotein absence in Canadian wheat cultivars may result from the ancestral cultivars Thatcher and Prelude that carry the mutation at GBSS1 B locus.

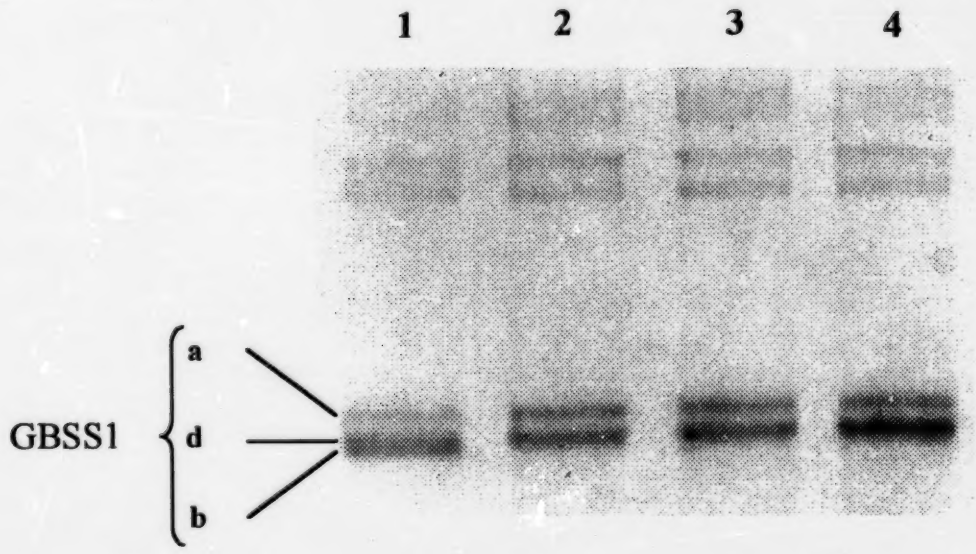

Figure 1: SDS-PAGE separation of GBSS1 proteins isolated from wheat starch granules. Lane1: AC Reed (wild type); Lanes 2-4 Columbus, AC Minto and AC Barrie, null GBSS1B. Arrow indicates the position of GBSS1A (a), GBSS1B (b) and GBSS1D (d).

Table 1
Distribution of GBSS1 isoproteins in Canadian wheat cultivars.

GBSS1 proteins	Canadian Market Class	Cultivars
Normal	Canada Western Red Spring (CWRS)	AC Eatonia, Apex, Benito, CDC Teal, Huron Leader, Marquis, Park, Red Bob's 222, Redman, Ruby.
	Canada Prairie Spring (CPS)	AC Crystal, AC Foremost, AC Karma, AC Taber, AC Vista, Biggar, Cutler, HY320, Genesis.
	Canada Western Red Winter (CWRW)	AC Readymade, CDC Kestrel, Winalta.
	Canada Western Extra Strong (CWES)	Bluesky, Glenlea, Laser, Wildcat.
	Canada Eastern Red (CER)	AC Baltic, AC Drummond, AC Mimi, AC Pollet, Belvedere, Casavant, Consens, Messier.
	Soft White Spring (SWS)	AC Reed.
	Soft White Winter	Harus
	Not in any Class	AC Walton, Aquino, Early Red Fife, Hard Red Calcutta, Laval19, Preston, Red Fife, Stanley, CDC Bavaria (Spelt wheat).
Null GBSS1 B	Canada West Red Spring (CWRS)	AC Barrie, AC Cadillac, AC Cora, AC Domain, AC Elsa, AC Intrepid, AC Michael, AC Minto, AC Splendor, , Canthatch, CDC Makwa, CDC Merlin, Chester, Columbus, Conway, Garnet, Invader, Katepwa, Kenyon, Laura, Manitou, McKenzie, Napayo, Neepawa, Pasqua, Pembina, Prelude, Prodigy, Regent, Rescue, Reward, Roblin, Saunders, Selkirk, Sinton, Thatcher
	Canada Eastern Red (CER)	AC Brio, AC Voyageur, Pacific.
	Canada Western Red Winter (CWRW)	Norstar

6.2 Purification of GBSS1 D Isoprotein, Antibody Production and its Cross Reactivity with other Plant GBSS1

Purification of the GBSS1 D isoprotein by electroelution revealed a single polypeptide band as reported previously (Final Report, ADF Project 9400081). All other starch granule proteins observed in the whole protein sample were not present in the six purified samples of GBSS1 D. This suggests that we have purified the D genome encoded GBSS 1 to apparent homogeneity. Immunoblot analysis revealed that the antiserum raised against the GBSS1 D isoprotein recognized all three GBSS1 isoproteins (A, B and D) from wheat kernels (Fig. 2). The pre-immune serum did not show any reaction with the starch granule polypeptides.

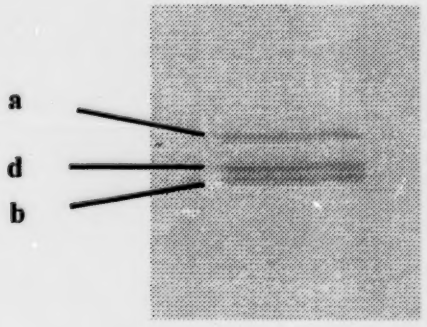

Figure 2. Immunoblot analysis to show the reactivity of GBSS1 D antibodies with GBSS1 A (a), B (b) and D (d) isoproteins.

The electrophoretic separation of GBSS1 from different cereals, pea and potato is shown in Fig. 3. Wheat genotypes AC Reed and *Triticum spelta* (Fig. 3 lanes E and F) revealed two polypeptides (A, the slow migrating and B, D relatively faster migrating). *Triticum monococcum* Fig. 3 lane G) is a diploid wheat presumed to be the 'A' genome donor (Dvorak et al., 1988) as expected showed the presence of a single GBSS1 polypeptide. All the other cereals also showed a single GBSS1 polypeptide. The GBSS1

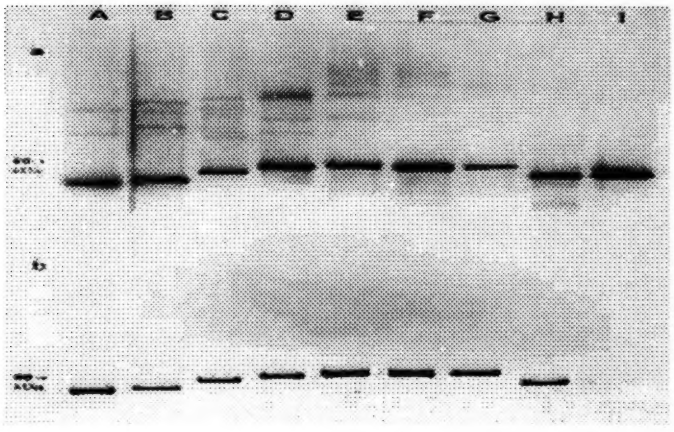

Fig. 3 Analysis of starch granule proteins by denaturing gel electrophoresis (a) and immunoblot analysis (b) with GBSS1 D antibodies. A=corn, B=rice, C=oat, D=barley, E=wheat (AC Reed), F=wheat (*T. spelta*), G=*T.monococcum*, H=pea and I=potato.

from different plants had different molecular weights. The molecular weight of GBSS1 from barley and wheat is higher than that of corn rice, oat, pea and potato (Fig. 3 a & b). The molecular weight of mature GBSS1 of cereals, pea and potato is in the range of 58 to 60 kDa.

Immunoblot analysis indicates that the GBSS1 antibodies also reacted with GBSS 1 from major cereals and dicot plants studied. The cross reactivity of GBSS1 antibodies is expected because of the more than 60% amino acid sequence similarity between GBSS1 from plants studied in this experiment. However, the GBSS1 antibody did not recognize other starch synthases or biosynthetic enzymes present in starch granules. The GBSS1 antibodies can be used to study the role of GBSS1 in starch biosynthesis.

6.3 Identification of null B and D GBSS 1 mutant SDS-PAGE Analysis and N-Terminal Sequencing of GBSS1 A Isoprotein.

Wheat germplasm evaluation for GBSS1 mutants has been described before (ADF Project Report 9400081; Demeke et al., 1997; 2000). More than 300 wheat genotypes were screened and resulted in the identification of null A, B and D genomes or double mutants which lack GBSS1 A and B isoproteins (Demeke et al., 1997). We previously had identified a GBSS1 null B and D wheat line while screening F2 seeds (K107 x Bai Huo) for waxy endosperm starch (Chibbar et al 1997). The seeds from this line

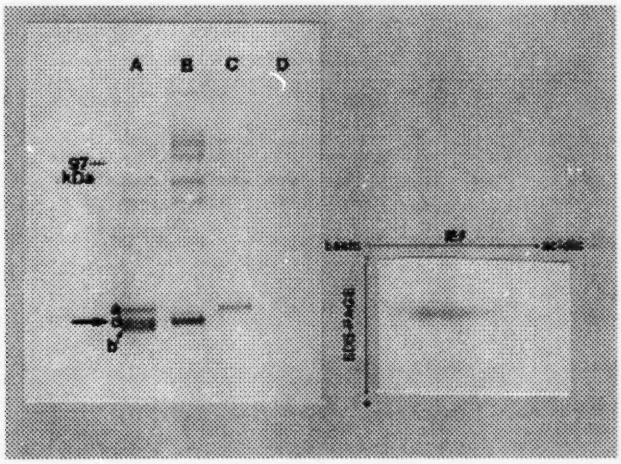

Figure 4. Identification of proteins associated with starch granules extracted from the endosperm of wild, partially waxy, and waxywheat lines. Left: SDS-PAGE. Lanes A-D: AC Reed (wild); PI235238 (null A and B); CDCW6 (null B and D); and waxy line. Arrow indicates location of the GBSS1 isoprotein (GBSS1 A [a], GBSS1 B [b], and GBSS1 D [d]. Right: Isoelectric focusing followed by SDS-PAGE for CDCW6 GBSS1 isoprotein. Note that a single protein with three subunits is seen.

(CDCW6) gave a reddish-brown color with blue spots in the centre of the endosperm when stained with iodine. Comparison of the CDCW6 GBSS1 isoprotein profile with wild, PI235238, and

waxy isoproteins indicated CDCW6 to have only the GBSS1 A isoprotein (i.e., null B and D for GBSS1, Fig. 4, left). Two-dimensional gel-electrophoresis showed the presence of a single GBSS1 isoprotein for CDCW6 (Fig. 4, right). The fully waxy type lacked all three GBSS1 isoproteins as expected, and PI235238 lacked the null A and B GBSS1 isoproteins.

N-terminal protein sequencing was performed to confirm if the CDCW6 isoprotein was granule bound starch synthase by comparison with the amino acid sequences of GBSS1 A isoproteins from *T. monococcum* and *T. urartu* (Table 2). Several earlier reports have indicated that *T. monococcum* is the A genome donor for hexaploid wheat, but in a recent study, *T. urartu* has been proposed to be the A genome donor (Dovorak 1998). There was a high degree of amino acid sequence similarity among CDCW6, *T. monococcum* and *T. urartu*, indicating the CDCW6 isoprotein to be GBSS1. However, there was a unique difference at position four, in that CDCW6, *T. urartu* and *T. monococcum* had cysteine, glycine and serine, respectively. *T. monococcum* also had a glycine instead of glutamic acid at position 15, in contrast to the amino acid sequence reported for *T. monococcum* by Taira et al (1995).

TABLE 2

N-Terminal Amino Acid Sequences of GBSS1 A Isoproteins

GBSS1	Source	Protein sequence
A	*T. monococcum*	A-T-G-**S**-G-G-M-N-L-V-F-V-G-A-**G**-M-A-P-V?
A	*T. urartu*	A-T-G-**G**-G-G-M-N-L-V-F-V-G-A-E-M-A-P[a]
A	CDCW6 (T. aestivum)	A-T-G-**C**-G-G-M-N-L-V-F-V-G-A-E-M-A

[a]The N-terminal protein sequence of *T. urartu* is from Fujita et al (1996). The letters in bold indicate unique amino acids.

6.4 Amylose Concentration of Waxy and Partially Waxy Wheat Lines

The amylose concentration of wheat endosperm starch has been reported to vary depending upon the method used in its determination. For example, the two wheat lines crossed to produce waxy wheat, K107 (null A and B for GBSS1) and Bai-Huo (null D for GBSS1) have been reported to have amylose concentrations varying from 16-25% (Hayakawa et al 1997, Kiribuchi-Otobe et al 1997, Muira and Tanii 1994, Nakamura et al 1995) and 24-30% (Hayakawa et al 1997, Yamamori et al 1995), respectively. Some of the methods which have been used to determine amylose concentration include: colorometric determination of iodine complexed with amylose, also known as blue value (Knutson 1986, Knutson and Grove 1994, Yasui et al 1996), potentiometric iodine titration (Adkins and Greenwood 1966, Banks et al 1974), autoanalyzer (Nakamura et al 1995), gel-filtration chromatography (Denyer et al 1995), and high performance size-exclusion chromatography (Bradbury and Bello 1993). Each method is limited in its ability to accurately determine amylose content. Recently, Gibson et al (1997) reported a procedure where Con A is used to complex and selectively precipitate amylopectin, thereby, allowing the determination of amylose. Using this method, we found significant differences in the amylose concentrations of waxy, CDCW6, PI235238, and AC Reed (Table 3). The CDCW6 and PI235238 lines contained approximately 12% and 23% amylose, respectively (Table 3). The 23% amylose concentration of PI235238 starch was similar to that of K107 starch (null A and B) as reported by Nakamura et al (1995). These results suggest that mutations at the GBSS1 A and B loci did not substantially reduce the amylose concentration in wheat starch. It has also been reported that mutation at the B locus reduces amylose concentration by more than 3%, while mutation at either

the A or D locus reduces amylose concentration by less than 2% (Miura and Sugawara 1996) as compared with wild type wheat starch. Our results showed that a combination of null B and D GBSS1 alleles significantly reduced the amylose concentration as compared with the null A and B GBSS1 alleles. However, studies incorporating different lines of all three GBSS1 double mutants could further reveal effects of waxy A, B, and D isoproteins on amylose concentration. The waxy lines contained about 5% amylose, which was higher than the 0.5 to 2% reported by others (Nakamura et al 1995, Yasui et al 1996).

TABLE 3

Amylose Concentration of Waxy (W2), Partially Waxy (CDCW6, PI235238) and Wild (AC Reed) Wheat Starches

Lines	GBSS1 isoprotein	Amylose (%) Megazyme	HPSEC
CDCW6	A	12.4	12.7
Waxy 1	-	5.5	0.0
Waxy 2	-	5.5	0.0
PI235238	D	23.5	22.3
AC Reed	A, B, D	25.8	25.0
LSD .05		2.3	-
LSD .01		3.2	-

High-performance size exclusion chromatography was used to confirm the amylose concentration data obtained by precipitation with Con A (Fig. 5). In this procedure, grain starch was debranched by isoamylase and fractionated by size-exclusion on a high-performance liquid chromatography system. Under the experimental conditions, amylose and debranched amylopectin had retention times of approximately

11.0 and 16.0 min, respectively. The analysis showed no amylose for waxy wheat, 12.7% amylose for CDCW6, 22% amylose for PI235238, and 25% amylose for AC Reed (Fig. 5). Thus, the results were similar to those obtained with the Con A method of Gibson et al (1997), with the exception of waxy wheat starch (Table 3). In the case of waxy wheat starch, Con A might not have precipitated all of the amylopectin, thus giving a relatively high amylose concentration. It is also possible that amylopectin in waxy wheat might be less branched and contains lower molecular weight polymers that are not precipitated with Con A. However, this assumption needs to be confirmed by detailed analysis of starch structure. Our results suggest that high performance size-exclusion chromatography of debranched starch gives a more accurate estimation of amylose and amylopectin concentrations in grain starch than the Con A method.

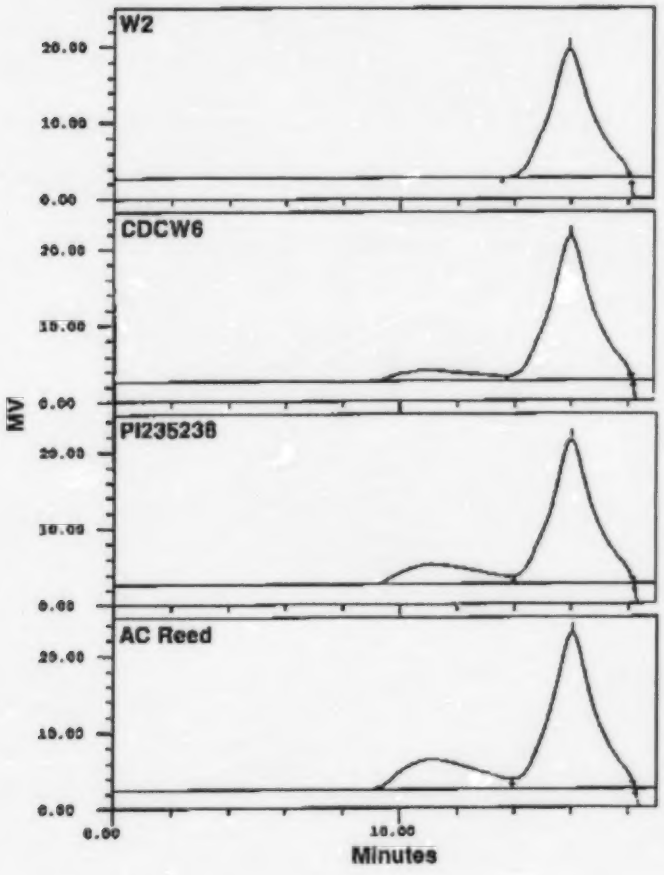

Figure 5: High-performance size exclusion chromatography (HP-SEC) analysis of debranched starch isolated from waxy, partially waxy, and non-waxy wheat lines. Waxy, CDCW6, PI235238, and AC Reed contained 0, 12.7, 22.3 and 25% amylose, respectively.

6.5 Thermal Transition Temperature of Waxy and Partially Waxy Wheat Lines

The data for transition temperatures (T_o, T_p, and T_c) and enthalpy (ΔH) is summarized in Table 4. Grain starches extracted from waxy, CDCW6 and PI235238 wheat lines exhibited relatively high thermal transition temperatures. There were significant ($P = 0.05$) differences among the onset and peak temperatures, as well as in the enthalpies of gelatinization. The onset and peak temperatures for waxy, CDCW6, and PI235238 were

higher than corresponding values for AC Reed (wild type). Starch extracted from waxy, CDCW6, and PI235238 also had higher enthalpies of gelatinization than did AC-Reed.

TABLE 4

Thermal Transition Temperatures and Enthalpies of Waxy (W2), Partially Waxy (CDCW6, PI235238) and Wild (AC Reed) Wheat Starches

	Endothermic transition temperatures (°C)				
Starch Samples	Onset (T_o)	Peak (T_p)	Conclusion (T_c)	Range ($T_C - T_o$)	Enthalpy of gelatinization (ΔH, J/g)
W2	55.6	63.5	73.5	17.9	10.3
CDCW6	56.0	66.3	75.8	19.8	11.0
PI235238	56.4	64.0	72.2	15.8	10.4
AC Reed	51.9	60.8	71.4	19.5	9.2
LSD 0.05	0.54	0.49	NS*	NS	0.99
LSD 0.01	0.89	0.81	NS	NS	NS

* NS = Non significant

Waxy wheat starch requires a large amount of energy for gelatinization (Fujita et al 1998), and thus has a high enthalpy of gelatinization, as confirmed by our study. Hayakawa et al (1997) also has reported higher transition temperatures and enthalpies of gelatinization for waxy wheat starch than for non-waxy wheat starch. Significant variations in the onset of gelatinization were also observed in starches of partially waxy and non-waxy wheat cultivars (Zeng et al 1997). Information on molecular weight,

degree of polymerization and branching of waxy, partially waxy and wild wheat starch would further elucidate the differences in thermal and pasting characteristics.

6.6 X-Ray Diffraction Analysis of Waxy and Partially Waxy Wheat Lines

The four starch samples were all determined to be that of the "A" type polymorph, which is common for cereal starches (data not shown). The relative peak intensities in X-ray diffraction data indicate the crystallinity of starch granules (Abdel-Aal et al 1997).

TABLE 5

Percent Relative Intensity[a] of Major Peaks in the X-Ray Diffraction Patterns of Waxy (W2), Partially Waxy (CDCW6, PI235238) and Wild (AC Reed) Wheat Starches

	X-ray diffraction angle (2θ)			
Starch sample[b]	15.2 (5.8)[b]	17.2 (5.2)	18.0 (4.9)	23.2 (3.8)
W2	27.8	36.7	36.7	36.7
CDCW6	25.8	30.8	30.8	30.0
PI235238	19.3	28.9	29.8	28.9
AC Reed	16.9	20.2	20.8	20.2

[a] Starch peak intensity/standard peak intensity x 100.

[b] Numbers in parentheses are starch crystal d-spacing (Å).

Waxy wheat starch had the highest crystallinity followed by CDCW6, and wild wheat starch had the lowest crystallinity (Table 5). The high crystallinity of waxy wheat starch results from its high amylopectin content (Fujita et al 1998, Hayakawa et al 1997). In addition, the X-ray data was negatively associated with amylose content as determined by HPSEC (Table II), in that the lower the amylose content, the higher the crystallinity, and vice versa.

6.7 Fully Waxy Wheat Breeding Lines

The first cross between a waxy wheat prototype and Canadian parents was made in 1995. The most advanced waxy wheat lines were advanced via single seed descent (SSD) in the greenhouse. In excess of 200 lines were rowed out in 1997 and selected for early maturity, shattering resistance and lack of hybrid necrosis. These lines (n=34) were grown in a replicated trial (n=2) at a single location in 1998.

TABLE 6
Agronomic performance of waxy wheats in 1998.

		Grain Yield %	DSE d	DPM d	Ht. cm	TWT kg/hL	Kwt. mg	Bunt %
1	Katepwa	80.1	-2.0	-4.0	26.0	0.0	0.0	0
2	Foremost	100.0	0.0	0.0	0.0	0.2	6.0	-17
3	W2	86.6	0.5	1.5	14.0	-3.2	-6.7	-17
4	98.11	103.1	0.5	-1.0	19.5	-1.1	1.6	-15
17	98.14	105.1	-0.5	-1.0	10.0	0.7	-3.3	23
20	98.17	109.6	0.5	0.0	9.0	1.3	-3.2	-1
22	98.19	105.9	1.0	1.0	6.0	0.6	-2.2	15
26	98.23	102.6	-1.5	-1.0	6.5	-1.9	8.7	-3
31	98.28	101.5	-1.0	-0.5	11.0	-0.3	-2.1	-17
33	98.30	102.9	0.5	0.0	10.0	-2.0	-2.1	-25

DSE = days to spike emergence, DPM = days to physiological maturity, Ht. = plant height
TWT = grain test weight, KWt = kernel weight, Bunt = common bunt infection.

The grain yield data is expressed as a % of Katepwa. The remaining traits are expressed as a deviation. Lines that were not advanced to the final stage of quality evaluation are not included in the tables for the sake of brevity. The agronomic performance of seven lines was satisfactory compared to the check cultivars Katepwa and AC Foremost. Some of the lines yielded up to 10% higher than AC Foremost and 25% higher than the waxy prototype 'W2'. However, many of the lines were small-seeded. All lines were susceptible to leaf and stem rust. The latter results prompted us to discontinue this

material and accelerate the advancement of lines from the first backcross to AC Foremost.

The lines from the first backcross to AC Foremost were advanced via SSD, tested in our rust nursery in 1998 (n= 360 lines) and the most resistant lines (n=41) were multiplied in our winter nursery. These lines were tested in a replicated trial at two locations in 1999. This second cycle of breeding material had much better rust resistance and resistance to common bunt. In addition the yield of the most promising lines was up to 20% higher than that of the check cultivars. The seed size of most lines was larger than that of Katepwa. These lines were confirmed as being fully waxy by Falling Number analysis. Based on these results, two lines were entered into the cultivar registration testing system in 2000. Since there is no official trial for this end-use quality of wheat a pre-coop test was deemed acceptable.

TABLE 7
Agronomic performance of waxy wheats in 1999.

	Grain Yield %	DSE d	DPM d	Ht. cm	LDG 1-9	LBL 1-9	TWT kg/hL	Kwt mg
1 Katepwa	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2 Foremost	102.5	-0.3	3.5	-28.3	-0.5	-0.5	-1.3	2.3
22 99020	102.5	0.5	2.5	-22.8	-0.5	-3.0	-2.0	0.5
23 99021	107.8	-0.5	3.3	-29.5	-0.5	-1.5	-1.6	2.5
26 99024	112.8	-1.5	3.5	-23.0	-0.5	-3.5	-0.7	6.5
27 99025	117.3	0.5	4.0	-17.3	0.0	-4.0	-1.4	-1.5
28 99026	109.4	-2.0	2.3	-14.8	-0.5	-4.0	0.0	4.3
29 99027	121.5	0.0	3.5	-19.8	-0.5	-3.5	-0.2	1.3
32 99030	105.1	-1.8	2.3	-16.5	-0.5	-4.0	-0.2	7.8
33 99031	104.2	-0.5	4.3	-28.0	-0.2	-3.5	-3.6	1.8
34 99032	110.1	2.8	6.0	-31.3	-0.5	-5.5	-3.3	-1.3
37 99035	107.1	-0.8	3.5	-23.0	-0.5	-2.5	-1.7	7.8
40 99038	104.8	1.5	4.8	-27.3	-0.5	-4.5	-3.6	-0.3
42 99040	101.8	-1.0	2.5	-24.0	-0.5	-3.5	-2.6	-0.5

DSE = days to spike emergence, DPM = days to physiological maturity, Ht. = plant height LDG = lodging, LBL = leaf blight, TWT = grain test weight, KWt = kernel weight, Bunt = common bunt infection.

In 1997 we initiated backcrossing of the waxy trait into the CWRS quality cultivar CDC Teal and into CWES quality parental material. We have now reached the BC5 stage and are lining out material from the lower levels of backcrossing. In addition we have made 3 further backcrosses to CPSR parents.

6.8 Partially Waxy Wheat Breeding Lines

The first populations aimed at the designed to develop partially waxy wheat with Udon noodle quality were initiated in early 1995. This material was delayed in its development somewhat by the loss of the California winter nursery to the Karnal bunt scare in early 1996. None-the-less lines were sent to our winter nursery in 96/97 after planting remnant seed from our 1995 summer nursery. In excess of 300 lines were tested in a series of replicated (n=2) trials at a single site in 1997. Quality analyses (protein, starch swelling

TABLE 8
Agronomic performance of selected partially waxy wheats (1997-1999)

		Grain Yield %	DSE d	DPM d	Ht. cm	LDG 1-9	TWT kg/hL	Kwt. mg	Bunt %
1	Katepwa	80.9	*	*	15.0	0.6	*	*	8
2	Karma	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0
3	Reed	108.2	-1.3	-0.2	-4.3	-0.5	-0.6	-3.5	47
4	Eradu	91.7	-1.4	0.2	-6.5	0.0	-0.9	-1.1	21
5	Cadoux	75.9	-1.1	0.0	-9.9	0.5	-2.1	-0.3	21
6	W97274	93.7	-3.0	-0.3	-3.2	0.5	0.8	-2.2	62
8	W97284	95.9	0.3	1.0	2.0	0.0	-1.9	-3.4	55
9	W97386	94.9	1.1	1.0	2.1	0.0	-1.5	-1.9	40
12	W97476	98.3	-1.5	-0.8	-6.1	0.7	-1.7	-6.3	61
15	W97526	99.8	-2.3	-2.7	9.2	0.0	1.7	-2.5	44
16	W97542	94.5	-0.3	-1.1	9.7	-0.2	0.5	-2.9	57

DSE = days to spike emergence, DPM = days to physiological maturity, Ht = plant height
LDG = lodging, TWT = grain test weight, KWt = kernel weight, Bunt = common bunt infection.

and dough mixing properties) were conducted on 100 of the lines. Selected lines were evaluated for viscosity at the Grain Research lab by Dr. J. Kruger. Of these lines 31 were retested in 1998 and 12 in 1999. These lines were also tested in order to confirm that they carried the desirable waxy allele that reduces amylose content by a few percentage points.

A number of lines were higher yielding than the checks Eradu and Cadoux in addition to having a lower protein content. Due to the genetic background of this material, the rust and common bunt resistance will need to be improved in further breeding efforts. Two lines were selected for cultivar registration trials in 2000. Six of these lines were tested at the Grain Research Lab by Dr. D. Hatcher for noodle quality in 1999. The two lines selected for further testing were the most promising after undergoing a full battery tests.

In addition to the above lines we tested in excess of 300 partially waxy lines in unreplicated yield trials in 1998 and over 150 lines in 1999. These lines were selected from rust resistant material identified in 1997 and 1998.

In excess of 19,000 lines of potential partially waxy wheat were evaluated in rust nurseries between 1997 and 1999. The vast majority of this material had to be dropped due to inadequate rust resistance. New populations were initiated in 1997 to address this shortcoming. The first DH (doubled haploid) lines with improved disease resistance are being yield-tested in 2000.

6.8 Single Seed Descent Populations

The single seed descent method is used to generate homozygous lines as rapidly as possible with the least expense incurred. Out of 13 populations initiated in 1995, eight were advanced to the F6 and 5 to F5 in early 1997. These lines were multiplied in the

field in 1997 and grown in replicated single row trials at two sites in 1998. Starch was extracted from 192 lines grown in 1998 and the following GBSS1 nulls were identified: A, B, D and AB and ABD. A subset of these lines, representing the different null types was re-tested in the field in 1999. The ultimate goal is test this material for amylose content in order to determine the stability of starch composition over growing environments. The equipment necessary to perform this analyses broke down repeatedly and we have been unable to analyze the replicated grain samples.

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ITEM "B". Deviations from original workplan – extension of project by 14 months due to Dr. Demeke's departure in early 1999, to take other employment opportunities and difficulties in finding a replacement for him.

ITEM "C". Development of adapted fully waxy lines for cultivar registration purposes. Development of partially waxy lines with Udon noodle-making quality.

ITEM "D". Equipment-NIL.

ITEM "E". Personnel -

Dr. T. Demeke PRA (July 1, 1996 – July 31, 1998)-100 %

(August 1, 1998 – January 15, 1999) – 20%

Dr. G. Sreeramulu PDF (November 1, 1999 – March 31, 2000) – 50%

C. Miskolczi (Tech. Asstt.) (July 1, 1996 – August 31, 1996)-100 %

D. Stevenson (Tech. Asstt.) (June 29, 1998 – April 29, 1999) –100%

B. Bjarnarson (Tech. Asstt.) (May 1, 1999 - August 31, 1999) – 100%

C. Rathnayaka (Tech. Asstt.) (March 1, 2000 – May 30, 2000) – 50%

Other contributors:

P. Hucl (July 1, 1996 – June 30, 2000) -5%

R. Chibbar (July 1, 1996 – June 30, 2000) -5%

ITEM "F". Expense statement - To date to be sent under separate cover.